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Chromatographic Determination of Doxycycline Using Three New Reversed Phase Columns

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UPLC-like method, Doxycycline, Fast LC Analysis, Fused core, luna, monolithic columns.

ABSTRACT
In the present study, a rapid, simple and economical reversed phase HPLC method has been developed for separation of Doxycycline from its therapeutically active impurities (Oxytetracycline and Methacycline). The samples were separated isocratically on three different new reversed phase C18 columns: Ascentis Express C18 column 10 cm x 4.6 mm, 2.7µm (Sigma Aldrich), Luna 2.5 µm C18 column and Onxy Monolithic C18 column 50 mm x 4.6 mm (Phenomenex). Isocratic potassium dihydrogen phosphate (0.005 M) pH=5 containing 0.2 g/L of sodium edetate (solvent A) and acetonitrile (solvent B) 80:20, v/v was used as a mobile phase at a flow rate of 0.5 mL/min under ambient conditions using UV detection at 254 nm. The Doxycycline was separated from its impurities Oxycycline and Methacycline in less than 10 min using fused core column and in less than 5 min using monolithic and luna columns. The linearity of the method was calculated to R^2 = 0.9998, 0.9999 and 0.9999 for luna, monolithic and fused core columns respectively over a concentration range of 0.07- 0.3 mg/mL. The limit of detection (LOD) was found to be 0.041, 0.030 and 0.012 µg/mL for luna, monolithic and fused core columns respectively. The limit of quantitation (LOQ) was found to be 0.120, 0.080 and 0.040µg/mL for luna, monolithic and fused core columns respectively. Additionally, the developed HPLC method showed an acceptable value of repeatability and intermediate precision. Furthermore, the asymmetrical factor for Doxycycline peak, resolution and number of theoretical plate for the three different columns were calculated. The simplicity and validity of the method makes it highly reliable and suitable for analysis of Doxycycline.

Introduction

Doxycycline- Monohydrate (DOX)

DOX,1-dimethylamino-2,4a,5,7,12-pentahydroxy-11-methyl-4,6-dioxo-1,4a,11, 11a,12,12a-hexahydrotetracene-3-carboxamide (Figure 1), is a semi-synthetic broad spectrum antibiotic obtained from oxytetracyline (OTC) and it can exist in two forms DOX monohydrate and DOX hyclate. Metacyline (MTC) is an intermediate of the synthetic pathway. During the transformation of MTC into DOX, some 6 – epidoxycycline (6-EDOX) can also be formed. Upon storage of DOX in solution, it may epimerize to 4 – epidoxycycline (4-EDOX). For this compound, keto-enol tautomerism between C11 and C12 occurs (Naidong et al., 1993). 4,6-Epidoxycycline (4,6-EDOX), the epimer of 6-EDOX, is a derivative of minor importance. 2-Acetyl-2-decarboxamidodoxycycline (ADDOX) can be present as a result of the presence of its analogue in the starting material used for synthesis. Usually DOX is used in human and veterinary medicines or as feed additive, due to its activity against a wide range of Gram-positive and Gram-negative pathogens. (Joshi and Miller, 1997).

4,6-EDOX	CH_3	H	$N(CH_3)_2$	H	$CONH_2$
4-EDOX	H	CH_3	$N(CH_3)_2$	H	$CONH_2$
6-EDOX	CH_3	H	H	$N(CH_3)_2$	$CONH_2$
ADDOX	H	CH_3	H	$N(CH_3)_2$	$COCH_3$
MTC		$=CH_2$	H	$N(CH_3)_2$	$CONH_2$
OTC	OH	CH_3	H	$N(CH_3)_2$	$CONH_2$
DOX	H	CH_3	H	$N(CH_3)_2$	$CONH_2$
	R^1	R^2	R^3	R^4	R^5

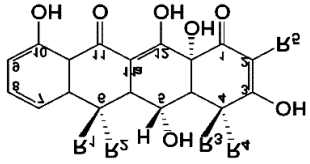

Figure 1 Structures of DOX and its impurities.

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The chemical structures of DOX and its related substances are shown in figure 1 The potential impurities are therapeutically inactive except OTC and MTC. Their content in commercial bulk DOX is restricted. The Pharm. Eur. sets the limits for 6-EDOX to be not more than 0.5% and any other impurity to be not more than 2% (Pharmacopoeia, 2008).

In the literature, TLC methods were established for determining of DOX (Choma et al., 1999, Oka et al., 1994).

Also, CE methods were used for the purity control of DOX and its impurities have been reported (Van Schepdael et al., 1995, Castellanos Gil et al., 2000). With the CE method described (Castellanos Gil et al., 2000), the above mentioned compounds are separated from each other including ADDOX, which is separated right after the main compound DOX.

LC methods have been described for the separation of DOX and its impurities (Dihuidi et al., 1985, Pharmacopoeia, 2005, Weng et al., 1990) using RP polymer based stationary phases, but these methods are unable to completely separate ADDOX from DOX. DOX was analysed on conventional columns by (Lu et al., 2004, Charoenraks et al., 2004, Castellanos Gil et al., 2000), also RP18- 25 mmx 4.6 mm monolithic column was applied for determination of DOX (Šatínskýa et al., 2005).

Recently, monolithic column was used as a stationary phase for the analysis of DOX. However, the elution was based on gradient system which has some drawbacks as the long time needed for column (re-) equilibration, limited choice of detectors, base-line drift on varying the eluent, lower signal-to-noise and signal-to-background ratios, spur peaks (impurities in weak eluent) and increased instrument complexity (Katz, 2002),(Cristofani et al., 2009).

Also, the monolithic stationary phase was used for the packing of the precolumn of solid phase extraction system of mixture of DOX and other tetracyclins (Sun et al., 2009).

Therefore, UPLC-like method with simple isocratic elution system was developed to separate and quantitate the OTC and MTC in the bulk DOX sample at very small quantity which is 1% of the DOX sample. The study was included a comparison between the monolithic, luna and a new stationary phase (Fused core) in order to develop the optimum method of analysis.

Experimental

Material and solutions

All chemicals and reagents used were of analytical reagent grade. They included Doxycycline - H_2O 95.9% (DOX) provided by Salutas Pharma GmbH, Oxytetracycline (OTC) provided by Augsburg, Germany, and Metayccline (MTC) provided by Dr. Ehrenstorfer GmbH. Reagents used included Potassium hydrogen phosphate supplied from Merck, Darmstadt, Germany, Disodium edetate. 2 H_2O supplied from Sigma-Aldrich, USA, and Acetonitril HPLC grade supplied from Baker, Deventer- Holland. Luna C18 column (50 x 3.00 mm, 2.5 µm) and Onyx monolithic RP-18 column (50 mm x 4.6 mm) were supplied from Phenomenex, while Ascentis Express C18 column (10cm x 4.6 mm, 2.7 µm) supplied from Sigma Aldrich.

Hundred milligrams of DOX or its impurities (MTC or OTC) were accurately weighed separately and quantitatively transferred to three different 100-ml volumetric flasks by dissolving each compound separately in small amount of mobile phase and complete to the volume of each flask with the mobile phase to obtain stock (1 mg mL^{-1}) solutions of each DOX, OTC, and MTC.

Working solutions: Serial dilutions for each compound were done to get three different drug concentrations 0.07, 0.1, 0.3 mg/ml.

Hundred micro liters of MTC and OTC working solutions were accurately transferred by micropipette to 10-ml volumetric flask and complete to the volume with DOX working solution to get a sample mixture of DOX, MTC, and OTC solution in the ratio 98:1:1 v/v/v respectively (0.7 µg/ml of MTC and 0.7µg/ml of OTC in DOX sample 0.07 mg/ml).

Accurately weighed 0.686 and 0.2 g of potassium dihydrogen phosphate and sodium edetat respectively were quantitatively transferred to 1- litter beaker by dissolving in small amount of distilled water and complete to 1- Litter with distilled water to get 0.005 M buffer with pH=5 (solvent A) . where the solvent B is acetonitrile HPLC-grade The mobile phase consists of a mixture of solvent A and B in the ratio 80:20 v/v. The mobile phase was degassed by sonication before use.

Apparatus

Merck Hitachi HPLC system

Interface:	D-6000 Interface (Merck/Hitachi)
Pump:	L-6200A Intelligent Pump (Merck/Hitachi)
Detector:	L-4500 UV-VIS (diode array) detector
Column oven	T-6300 (Merck/Hitachi)
Data analysed	D7000 HSM software (Merck/Hitachi)
Sensitive Scale	Horst Schirmer, Germany
Sonorex R K 100	W, Germany
Digital pH meter pH 522	WTW

Chromatographic conditions

The separation was performed on fused core, monolithic and luna columns with flow rate of 0.5 ml/min. All separations were performed at ambient temperature and 254 nm was the detection wavelength. Injection volume was 20 µl.

Results and Discussion

Development of the method

We applied the official HPLC method (Pharm.Eur., 2008) by using poly (styrene- divinylbenzene) (0.25 m, 4.6 mm, 8 µm at temp. 60°C) column for its determination however the obtained peak was very broad the drug's concentration was 0.8 mg/ml and the run time was 15 min . A good separation between DOX and its impurities "MTC and OTC" was obtained. The t_R of DOX, MTC and OTC were 11, 7 and 3 min respectively. Some problems were observed with the obtained chromatogram as peak broadening and tailing. A time variation in the baseline of a chromatogram observed.

The effect of the decrease drug concentration on its peak broadening was tested. A 70 µg/ml of the drug were injected to the HPLC system and all obtained peaks were still broad.

The effect of different packing material of the used analytical column on the peaks broadening was examined. Therefore, fused core, monolithic and luna columns were tested for that task.

In the case of fused core, the used mobile phase was phosphate buffer pH 8.0 Acetonitrile (80: 20) isocratic elution at flow rate (0.5 ml/min). The sample concentration for DOX was 100 µg/ml and the peak broadening was much better than that which obtained from the official method.

The effect of pH of the used mobile phase on the peak shape was tested. The same mobile phase with different pH was applied, alkaline, neutral and acidic. The peak sharpness was better at neutral pH than that obtained with alkaline; however, the baseline of the chromatogram was not constant.

The best conditions in terms of peak broadening, sharpness and baseline resulted at acidic mobile phase (pH=5). However, the

peak tailing still observed in the chromatogram.

A suggested mechanism for the observed peak tailing can be owned to the complexation between the drug and the packing material. Therefore a strong complexing agent was tried as ethylene diamine-tetra-acetic-acid (EDTA) in a concentration (0.2 mg/ml of solvent A) as a mobile phase additive and the peak tailing problem was completely solved. EDTA was suggested to replace the drug to liberate it without any effect on the t_R of the drug. EXtra peak was observed at t_R 3.0 min. This peak suggested being the drug epimer. This suggestion is documented with the fact of instability of DOX at pH of 6.0 and below, DOX undergoes conversion into its epimer (McCormick et al., 1957, Stephens et al., 1963).

A 70 µg/ml of the DOX sample containing 0.7 µg/ml of each MTC and OTC (i.e. each impurity 1% of the sample) were injected to the HPLC system with the last optimum conditions with flow rate=0.5 ml/min and a good separation was observed and all obtained peaks were symmetric, sharp without peak tailing.

The developed method was applied on other packing materials as luna and monolithic columns to test the method robustness (Figure 2).

With luna and monolithic column, the obtained peaks were sharp however the drug isomer's peak was not separated from the OTC's peak or almost poorly separated from each other. A trial to improve the resolution a decreasing in the floe rate to 0.3 ml/min was tested. A better peaks separation was observed with monolithic column however luna column still bad peaks resolution (Figure 3).

Another trial to improve the resolution for the luna by increasing the retention with decreasing the organic modifier percent was examined. Therefore, the acetonitrile was used as 15% of the used buffer v/v with flow rate (0.5 ml/min) and a much better separation was obtained (Figure 4).

The backpressure of different columns (luna, fused core, and monolithic) was tested at column length (50 mm) and flow rate (0.5 ml/min) for the analysis of DOX with new developed method. High backpressure was resulted with luna column due to the fine particle size followed with fused core column and the lowest backpressure was resulted with monolithic column which characterized with high permeability (Figure 5).

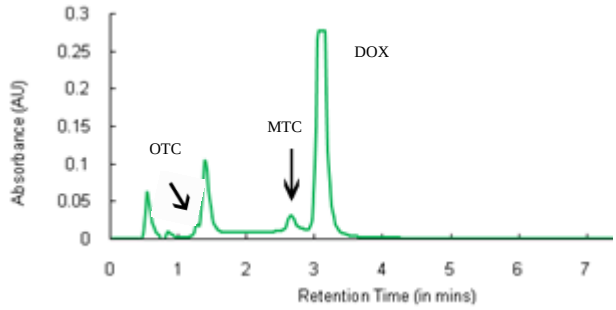


Figure 2: Representative chromatogram for the separation of DOX (0.07 mg/ml) from MTC (0.7µg/ml) and OTC (0.7µg/ml) by using the developed method, and A: fused core , B: monolithic, and C: luna columns.

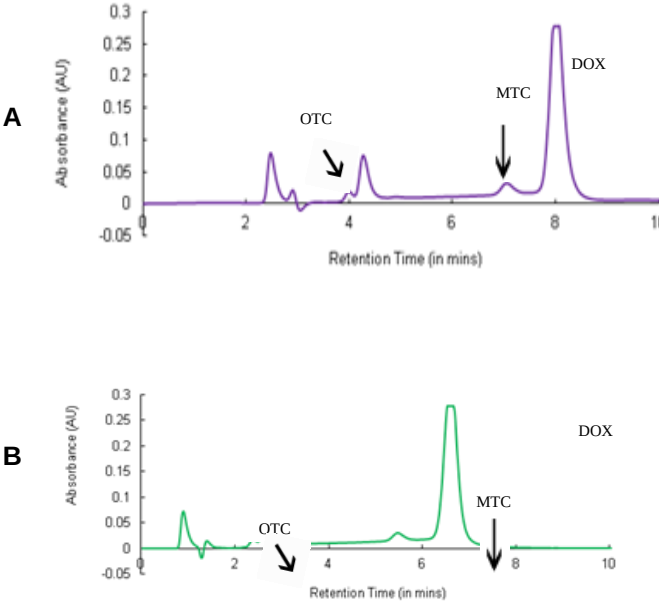
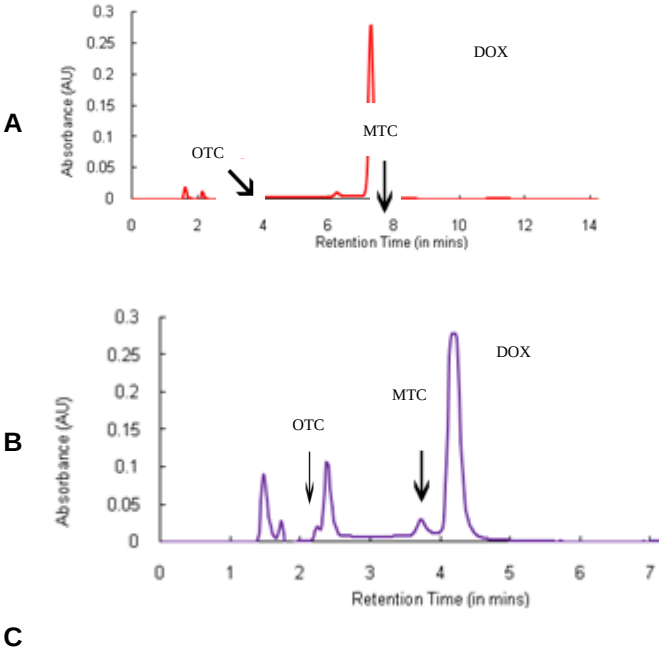


Figure 3: Representative chromatogram for the separation of DOX (0.07 mg/ml) from MTC (0.7µg/ml) and OTC (0.7µg/ml) by using the developed method, flow rate=0.3ml/min and, A: monolithic, and B: luna columns.



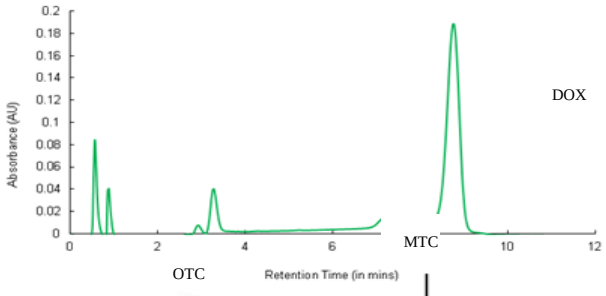


Figure 4: Represe ive chromatogram r the separation of DOX (0.07mg/ml). from MTC (0.7µg/ml) and OTC (0.7µg/ml) by using luna column with solvent A:B 85:15 v/v, flow rate=0.5ml/min.

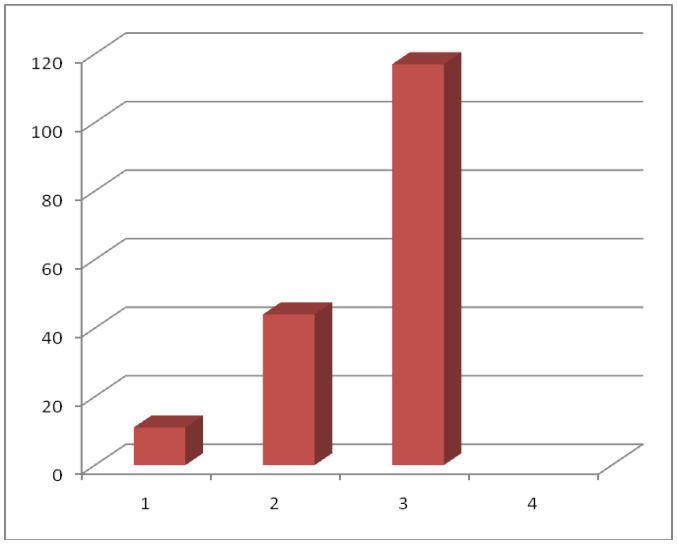


Figure 5: Plot of backpressure (bar) against different columns at column length (50 mm) and flow rate (0.5 ml/min) 1.Monolithic, 2. Fused core, and 3. Luna column.

Validation of the developed method: -

The new developed HPLC method for analysis of DOX was validated by:

Precision

To ensure assay precision within day repeatability (n=6) and between days repeatability (n=6) were assessed at 3 concentrations of DOX (Tables 1 and 2).

Table 1 : Within day repeatabilities of DOX on luna , monolithic and fused core columns over a concentration range 0.07-0.3 mg/ml using n=6.

Column type	Within day repeatability RSD% of AUC			Within day repeatability RSD% of t_R (n= 18)
	0.07	0.1	0.3	
	(mg/ml)			
Luna	0.62	0.74	0.70	0.51
Monolithic	0.57	0.66	0.71	0.53
Fused core	0.73	0.53	0.38	0.42

Table 2: between days repeatabilities of DOX on luna, monolithic and fused core columns over a concentration range 0.07- 0.3 mg/ml using n=6.

Column type	Between days repeatability RSD% of AUC			Between days repeatability RSD% of t_R (n= 18)
	0.07	0.1	0.3	
	(mg/ml)			
Luna core	0.60	0.81	0.79	0.54
Monolithic	0.65	0.70	0.80	0.52
Fused core	0.59	0.62	0.56	0.50

AUC= Area Under Curve, RSD% = Relative Standard Deviation , t_R = Retention time

Linearity, LOD and LOQ of the DOX

Calibration curve (peak area vs. concentration) for the analyzed DOX with new method was investigated over concentration range of 0.07- 0.3 mg/ml. The (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for the DOX (Table 3).

Table 3: Linearity, LOD and LOQ of DOX

Column type	LOD (µg/ml)	LOQ (µg/ml)	R^2
Luna	0.041	0.120	0.9998
Monolithic	0.030	0.080	0.9999
Fused core	0.012	0.040	0.9999

Limit Of Detection, LOQ= Limit of Quantitation, S /N =Signal- To- Noise Ratio LOD=
R^2 = Coefficient Of Determinationn

3.2.3. Performance parameters of the developed UPLC-like method:

Peak performance parameters were calculated according to fundamental equations (Table 4).

Table 4: Performance parameters for DOX on monolithic (50mm), luna (50mm) and fused core (100mm) columns at flow rate 0.5 ml/min.

Column type	Theoretical plate Number (N)	AF for DOX Peak	(Rs)DOX /MTC	Run time (min)
Monolithic	1841	1.1	1.5	5
Luna	486	1.2	1.1	3.5
Fused core	3775	1.1	2.5	8

Asymmetry Factor, AF= A/B At 10% Of Peak Height (A and B are tow half widths at each side of peak center)
Theoretical Plate Number, $N=16 (T_r/W)^2$
Resolution Value, $R_s = 2(T_{r2} - T_{r1} / W_{2+} W_1)$

Conclusion

Doxycycline one of the antibiotics which are widely used in treatment of different diseases. That is why pharmaceutical analysts are trying to develop different analytical methods for their determination.

Through the literature of the official and nonofficial HPLC analysis of the Doxycycline, conventional HPLC column was the only stationary phase which used for its analysis. There are many disadvantages for the use of that type of columns such as long analysis time. To the best of our knowledge there is no reported UPLC-like method for this drug. UPLC is a new type of fast HPLC technique which characterised by the short analysis time. Therefore, it was important to transfer HPLC method for analysis of Doxycycline to UPLC-like method by the use of new analytical columns which packed with more advanced stationary phases such as monolithic, luna and fused core columns. Moreover, comparisons were established among all selected stationary phases in terms of chromatographic run time, different flow rates, and the produced column back pressure. Performance parameters as AF, Rs and the theoretical plate number were also studied. The validation parameters (linearity, precession, LOD, LOQ, reproducibility) were therefore examined for the optimum condition. The aim of the work was to get the most suitable chromatographic method which can be used instead of the official HPLC method without affecting the resolution of peaks, sensitivity of the method or all validation parameters. Very short analysis time was obtained with all three columns.

In the official HPLC method of Doxycycline, the used temperature was 60°C not suitable for our lab work. However,

in the developed method, an ambient temperature was the optimum for the analysis.

The obtained column backpressure readings were watched at the flow rate 0.5 ml/min with column length (50 mm). Generally higher backpressure resulted with the use of luna because of the small particle size (2.5 µm dp). On using fused core column, the backpressure was reduced which attributed to the new technology in particles (2.7 µm) with a high-capacity and very pure porous silica layer which fused to a solid silica core. Monolithic column showed the lowest backpressure reading due to the high permeability of its bimodal structure.

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